Immunity, Vol. 3, 109–117, July, 1995, Copyright © 1995 by Cell Press

Specific Immunity to Listeria monocytogenes in the Absence of IFNγ

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Summary

Cytokine and cytokine receptor gene knockout mice provide powerful experimental systems to characterize the functions of these molecules in resistance to infectious disease. Such mice may also provide unique models of immune deficiency to learn whether manipulation of the immune response can overcome the specific dysfunction. We demonstrate that resistance of IFNγ gene knockout ($GKO^{-/-}$) mice to the intracellular bacterium Listeria monocytogenes is severely impaired compared with wild-type mice. However, immunization of $GKO^{-/-}$ mice with an attenuated L. monocytogenes strain generates antigen-specific CD8 T cell responses that can transfer immunity to naive hosts. Furthermore, vaccinated $GKO^{-/-}$ mice themselves exhibit 20,000-fold increased resistance to challenge with virulent L. monocytogenes and this resistance appears to be CD8 T cell mediated. These studies demonstrate that vaccination-induced immunity can overcome the absence of a cytokine that is critical for resistance to acute infection.

Introduction

Infection of mice with Listeria monocytogenes, a human bacterial pathogen associated with severe infections of immunocompromised individuals, has served as a model system for innate immunity and specific immunity to infectious disease (Kaufmann, 1993). L. monocytogenes is a facultative intracellular bacterium that infects macrophages and a variety of nonphagocytic cells, including hepatocytes (Cossart and Mengaud, 1989). The biology of L. monocytogenes infection has been characterized through the analysis of mutant organisms, attenuated for virulence in the mouse model, which exhibit specific defects in the pathogenesis of in vitro infection (Portnoy et al., 1992). L. monocytogenes enters the infected cell in a phagosome but escapes from this structure into the cytoplasm primarily through the actions of a secreted bacterial hemolysin, listeriolysin O (LLO). LLO^{-} mutants fail to escape from the phagosome and are avirulent (Cossart et al., 1989). Once in the cytoplasm of the eukaryotic cell, L. monocytogenes replicates and initiates movement through polymerization of host-derived F-actin, a process dependent on the L. monocytogenes Act A protein (Kocks et al., 1992). Act A mutant strains fail to polymerize actin in a polar fashion and are inhibited in cell–cell spread (Kocks et al., 1992; Brundage et al., 1993), which occurs when L. monocytogenes containing pseudopod-like structures are engulfed by adjacent cells. Such mutant strains exhibit approximately three orders of magnitude less virulence than wild-type strains (Brundage et al., 1993). Similarly, L. monocytogenes strains mutant in one or both genes encoding phospholipase activity have a reduced ability to spread between cells (Portnoy et al., 1992) and are attenuated for mouse virulence. This defect may result from the inability of phospholipase mutants to escape from double membrane structures formed during intracellular spread.

Innate or natural immunity to L. monocytogenes involves the recruitment and activation of phagocytic cells, including neutrophils and macrophages (Bancroft et al., 1991; Conlan and North, 1991). Treatment of mice with antibodies that deplete neutrophils (Conlan and North, 1994; Czuprynski et al., 1994) or prevent neutrophil extravasation (Rosen et al., 1989; Conlan and North, 1992) increases susceptibility to acute L. monocytogenes infection. Studies by the Unanue group in the scid mouse model have demonstrated that activation of macrophages after L. monocytogenes infection requires a complex set of interactions involving tumor necrosis factor-α (TNFα) and interleukin-12 (IL-12), produced by L. monocytogenes–infected macrophages (Tripp et al., 1993). These cytokines, in combination with bacterial factors, activate natural killer cells to produce IFNγ, which synergizes with TNFα to activate the microbicidal activities of macrophages (Wherry et al., 1991). This scenario is supported by demonstration of increased susceptibility to L. monocytogenes infection, of mice treated with monoclonal antibodies (MAbs) that deplete natural killer (NK) cells (Dunn and North, 1991a), neutralize TNFα (Bancroft et al., 1989) or IFNγ (Buchmeier and Schreiber, 1985), and is further supported by recent studies demonstrating increased susceptibility of TNFα type 1 receptor (Rothe et al., 1993) and IFNγ receptor (Huang et al., 1993) gene knockout mice. From these studies, cytokines such as IFNγ and TNFα have been implicated as major effector molecules in resistance to acute L. monocytogenes infection.

L. monocytogenes infection of mice elicits a full spectrum of specific cellular immune responses, including α–β T cell receptor (TCR)-expressing CD4 and CD8 T cells as well as γ–δ TCR-expressing T cells (Kaufmann, 1993). L. monocytogenes immune CD4 T cells and γ–δ T cells play a significant role in the generation and control of the granulomatous response to infection (Kaufmann, 1993) but are not required for the expression of acquired immunity to L. monocytogenes (Mielke et al., 1988; Mombaerts et al., 1993). In contrast, experiments using T cell subset depletion in vivo (Mielke et al., 1988, 1989; Dunn and North, 1991b) and specific T cell subset transfer (Bishop and Hinrichs, 1987; Lukacs and Kurlander, 1989), demon-

strate that CD8 T cells are effective mediators of acquired immunity to L. monocytogenes. It has been proposed that the cytoplasmic location of L. monocytogenes exposes the organism to the cytoplasmic MHC class I antigen presentation pathway, resulting in presentation of L. monocytogenes–derived peptides to CD8 T cells (Brunt et al., 1990).

The specific effector mechanisms employed by CD8 T cells in response to L. monocytogenes infection are not clearly defined. CD8 T cells possess the capacity to lyse target cells expressing the specific major histocompatibility complex (MHC) class I–peptide complex in vitro. In addition, CD8 T cells produce significant quantities of IFNγ and TNFα after antigen stimulation (Kaufmann, 1993). By analogy to natural immunity mediated by NK cell–derived IFNγ, it has been proposed that CD8 T cell–derived IFNγ-mediated activation of macrophages is the major pathway of specific immunity to infection. In contrast with this notion, we have presented evidence that L. monocytogenes–specific CD8 T cells can transfer immunity to naive mice in the presence of a MAb, which efficiently neutralizes mouse IFNγ in vivo (Harty et al., 1992). However, in vivo administration of cytokine neutralizing MAb does not ensure that all biologically relevant cytokine is effectively eliminated.

In the present experiments, we address the requirement for IFNγ as an effector molecule in natural and specific immunity to L. monocytogenes using mice with targeted disruption of the IFNγ structural gene ($GKO^{-/-}$ mice; Dalton et al., 1993). Our experiments show that $GKO^{-/-}$ mice are extremely susceptible to acute infection with virulent L. monocytogenes, demonstrating that IFNγ is a critical cytokine in natural resistance to L. monocytogenes infection. However, $GKO^{-/-}$ mice survive infection with an attenuated L. monocytogenes strain that primes an effective CD8 T cell response. L. monocytogenes immune CD8 T cells from $GKO^{-/-}$ mice, which are incapable of producing IFNγ, transfer antilisterial resistance to naive mice as effectively as CD8 T cells from L. monocytogenes immune mice that produce IFNγ. Strikingly, $GKO^{-/-}$ mice can be vaccinated with a single injection of attenuated L. monocytogenes and exhibit up to 20,000-fold increased resistance to virulent L. monocytogenes in the complete absence of IFNγ. These data demonstrate that IFNγ is not required for the development or expression of specific immunity to L. monocytogenes.

Results

$GKO^{-/-}$ Mice Are Extremely Sensitive to Infection with Virulent L. monocytogenes but Exhibit Wild-Type Resistance to *actA* Mutant L. monocytogenes

A role for IFNγ in resistance to acute infection with L. monocytogenes is supported by experiments demonstrating increased susceptibility of mice treated with a neutralizing anti-IFNγ MAb (Buchmeier and Schreiber, 1985) and mice with targeted disruption of the IFNγ receptor gene (Huang et al., 1993). In these reports, mice were challenged with normally nonlethal but relatively high levels of organisms (~0.1 LD_{50}, 10^3–10^4 colony-forming units [CFU]), which led to lethal infection when the functions of IFNγ were disrupted.

To obtain quantitative information on the susceptibility of mice with targeted disruption of the IFNγ structural gene ($GKO^{-/-}$ mice; Dalton et al., 1993) we infected H-2^d MHC expressing $GKO^{-/-}$, $GKO^{+/-}$, or BALB/c mice with graded doses of the virulent L. monocytogenes strain 10403s (Table 1). L. monocytogenes strain 10403s has a published LD_{50} of $10^{4.1}$ CFU in BALB/c mice (Bishop and Hinrichs, 1987). Consistent with this finding, 10403s infection of $GKO^{+/-}$ and BALB/c mice resulted in LD_{50}'s of greater than 10^4 CFU. These data demonstrate that the H-2^d MHC-expressing $GKO^{+/-}$ mice, generated by backcross to BALB/c, exhibit BALB/c-like resistance to L. monocytogenes and that the effects of IFNγ on survival in this system are independent of gene dosage.

In contrast, $GKO^{-/-}$ mice were extremely susceptible to virulent L. monocytogenes infection. The average LD_{50} of L. monocytogenes 10403s for $GKO^{-/-}$ mice, derived from the three independent experiments, was 10 CFU compared with greater than 10^4 CFU for $GKO^{+/-}$ and BALB/c mice. This IFNγ-dependent difference in resistance, demonstrates that IFNγ is a critical mediator of natural immunity to acute L. monocytogenes infection. Although not addressed in our studies, others have presented evidence that NK cells are the source of IFNγ in natural immunity to acute L. monocytogenes infection (Bancroft et al., 1991; Dunn and North, 1991a).

L. monocytogenes–encoded virulence factors, associated with distinct stages of the pathogenic infection, have been characterized through random transposon mutagenesis (Gaillard et al., 1986; Kocks et al., 1992) and directed mutagenesis (Brundage et al., 1993). To assess the role of IFNγ in natural immunity to L. monocytogenes infection, we have initiated experiments to characterize the virulence of defined L. monocytogenes mutants in $GKO^{-/-}$ mice. These experiments, employing mutant L. monocytogenes in conjunction with mice with a specific immune defect, may provide a powerful approach to the analysis of pathogenic infections and the functions of specific disrupted genes. Using this approach, we have determined that the attenuated L. monocytogenes mutant strain DP-

Table 1. $GKO^{-/-}$ Mice Exhibit Increased Susceptibility to Virulent L. monocytogenes Compared with $GKO^{+/-}$ and BALB/c Mice but Similar Resistance to *actA* Mutant L. monocytogenes

		LD_{50} (CFU)		
L. monocytogenes strain	Experiment	$GKO^{-/-}$	$GKO^{+/-}$	BALB/c
10403s (wild type)	1	$10^{0.9}$	ND	ND
	2	$10^{1.4}$	$10^{4.1}$	$10^{4.4}$
	3	$10^{0.7}$	ND	ND
DP-L1942 (*actA* mutant)	1	$10^{6.6}$	ND	$10^{6.9}$

Groups of 3–5 H-2^d MHC GKO–/–, $GKO^{+/-}$, or BALB/c mice were infected by intravenous injection with various numbers of virulent L. monocyotogenes strain 10403s or *actA* mutant strain DP-L1942. Actual numbers of CFU injected were determined by plating aliquots of relevant dilutions. Animals were observed for 14 days. ND, not defined.

L1942 (Brundage et al., 1993), which carries an in-frame deletion in the *actA* gene, exhibits an LD_{50} for GKO$^{-/-}$ mice that is similar to that seen with BALB/c mice (Table 1). The L. monocytogenes–encoded *actA* gene is involved in the process of actin polymerization and is necessary for cytoplasmic movement and cell–cell spread of the organism (Kocks et al., 1992). These data demonstrate that IFNγ is not required for resistance to L. monocytogenes that are incapable of cell–cell spread and suggest the hypothesis that the function of IFNγ in natural immunity to L. monocytogenes may inhibit this process.

Infection of GKO$^{-/-}$ Mice with *actA* Mutant L. monocytogenes Elicits Antigen-Specific CD8 T Cell Responses

The *actA* mutant L. monocytogenes strain DP-L1942 expresses the secreted virulence factor LLO and thus escapes from the phagocytic vesicle into the cytoplasm of the initially infected cell (Brundage et al., 1993). The organism then replicates in the cytoplasm but is unable to move inter or intracellularly. It has been suggested that the presence of L. monocytogenes in the cytoplasm of the infected cell is a requirement for presentation of L. monocytogenes antigens through the conventional MHC class I pathway, resulting in the activation of CD8 T cells (Brunt et al., 1990). Consistent with this notion, infection of wild-type mice with an *actA* mutant L. monocytogenes primes a CD8 T cell response that can transfer antilisterial immunity to naive hosts (Goossens and Milon, 1992).

To determine whether DP-L1942 infection of GKO$^{-/-}$ mice could prime a CD8 T cell response to L. monocytogenes, spleen cells from 7 day DP-L1942 infected GKO$^{-/-}$ or GKO$^{+/-}$ mice were restimulated in vitro under conditions used to generate CD8 T cell lines specific for the known L. monocytogenes CD8 T cell antigens, LLO (Pamer et al., 1991) and p60 (Pamer, 1994). LLO-specific CD8 T cell lines, from GKO$^{-/-}$ and GKO$^{+/-}$ mice, were generated by in vitro restimulation with P815 (H-2^d) cells transfected with the LLO gene as previously described (Harty and Bevan, 1992). p60-specific CD8 T cell lines were generated in a similar fashion using P815 cells transfected with the L. monocytogenes p60 gene as in vitro stimulators (Harty and Pamer, 1995). DP-L1942 infection of GKO$^{-/-}$ and GKO$^{+/-}$ mice generated CD8 T cells that could be restimulated in vitro to recognize either LLO- (Figure 1A) or p60- (Figure 1B) derived epitopes. Consistent with their origin, GKO$^{-/-}$-derived CD8 T cell lines are incapable of producing IFNγ after specific antigen stimulation (Figure 2). Thus, infection of GKO$^{-/-}$ mice with an *actA* mutant L. monocytogenes primed the CD8 T cell compartment and allowed the generation of antigen-specific CD8 T cells incapable of secreting IFNγ.

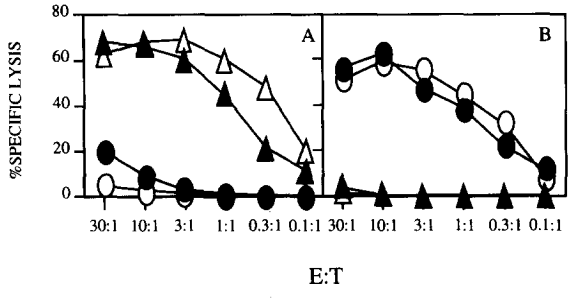

Figure 1. CD8 T Cells Specific for LLO and p60 Can Be Generated in the Absence of IFNγ

GKO$^{-/-}$ and GKO$^{+/-}$ mice were infected with DP-L1942 and 7 days later their spleen cells were restimulated in vitro with either PHem3.3, a LLO transfectant of P815 (A) or pP60.3, a p60 transfectant of P815 (B).

(A) LLO-specific CD8 T cell lines from GKO$^{-/-}$ (closed symbols) or GKO$^{+/-}$ (open symbols) specifically lyse P815 cells transfected with LLO (closed triangle, open triangle), but not p60-transfected P815 cells (closed circle, open circle).

(B) p60-specific CD8 T cell lines exhibit the opposite specificity.

CD8 T Cell-Derived IFNγ Is Not Required for Transfer of Antilisterial Resistance

We have demonstrated that CD8 T cell lines from L. monocytogenes immune H-2^d mice, specific for LLO (Harty and Bevan, 1992) or p60 (Harty and Pamer, 1995), can transfer significant levels of antilisterial immunity to naive mice. In another study, we demonstrated that the ability of LLO-specific CD8 T cell lines to transfer immunity was not inhibited by treatment of mice with a neutralizing anti-IFNγ MAb (Harty et al., 1992). Although the in vivo activity of the anti-IFNγ MAb was documented in these studies, it was impossible to rule out the possibility that biologically significant levels of IFNγ escape neutralization. In addition, this experimental approach does not identify the cellular source of IFNγ, which can be produced by NK cells, γ–δ, or CD4 T cells, as well as CD8 T cells.

To determine the requirement for CD8 T cell–derived IFNγ in adoptive transfer of antilisterial immunity, we analyzed the ability of LLO- and p60-specific CD8 T cell lines, from GKO$^{-/-}$ or GKO$^{+/-}$ mice, to transfer immunity to naive BALB/c hosts. The degree of immunity transferred was estimated by infecting groups of mice with ~10 LD_{50} of L. monocytogenes strain 10403s, in the presence or absence of L. monocytogenes–specific CD8 T cells and determining the number of L. monocytogenes in organ homogenates at 3 days postinfection. GKO$^{-/-}$-derived CD8 T cell lines specific for LLO (Figure 3A) and p60 (Figure

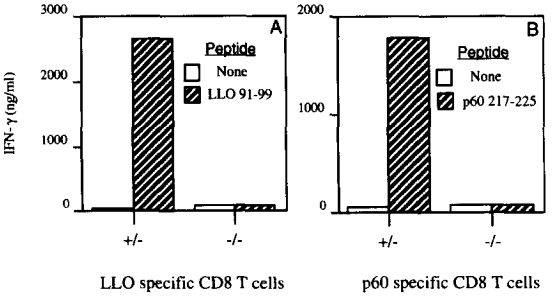

Figure 2. Antigen-Specific CD8 T Cells from GKO$^{-/-}$ Mice Cannot Produce IFNγ

LLO (A) and p60 (B) specific CD8 T cell lines from GKO$^{-/-}$ mice cannot produce IFNγ after antigen stimulation. In vitro peptide stimulation and IFNγ assays were performed as described (Harty et al., 1992).

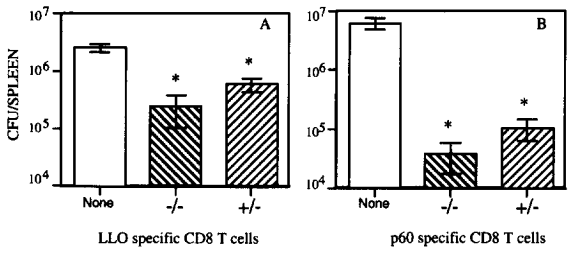

Figure 3. CD8 T Cell-Derived IFNγ Is Not Required for Adoptive Transfer of Immunity to L. monocytogenes

(A) LLO-specific (5 × 10^6) or (B) p60-specific (7 × 10^6) CD8 T cells from GKO$^{-/-}$ (left-to-right hatched bar) or GKO$^{+/-}$ (right-to-left hatched bar) mice were transferred to groups of naive BALB/c mice. These mice and controls (open bars) were challenged with 10 LD_{50} of 10403s. CFU/ spleen and liver were determined 3 days later. Results are pooled from two experiments with 6 mice per group and are expressed as mean ± SEM. Asterisk, $p < 0.005$ compared with control.

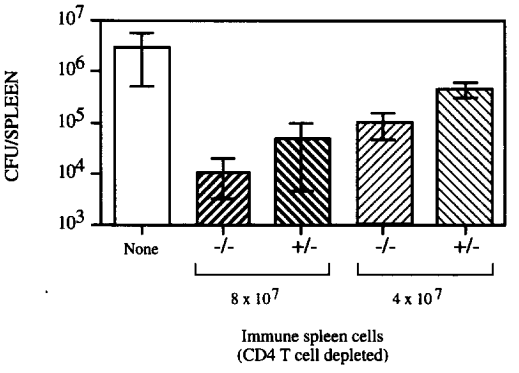

Figure 4. CD8 T Cell-Derived IFNγ Does Not Enhance Adoptive Transfer of Antilisterial Immunity

Spleen cells from GKO$^{-/-}$ or GKO$^{+/-}$ mice were obtained 7 days after infection with 4 × 10^6 CFU of DP-L1942. CD4 T cells were depleted with antibody and complement and either 4 × 10^7 or 8 × 10^7 cells were transferred to naive BALB/c mice. These mice and controls were infected with ~ 10 LD_{50} of 10403s. CFU/spleen and liver were determined 3 days later. Results are expressed as mean ± SD for groups of 3 mice.

3B) transferred significant immunity against L. monocytogenes infection, comparable to the degree of immunity transferred by GKO$^{+/-}$-derived CD8 T cells with the same antigen specificity. Transfer of BALB/c-derived CD8 T cells, specific for a non-L. monocytogenes antigen, results in no detectable immunity to L. monocytogenes in this assay (Harty and Bevan, 1992). Antigen-specific CD8 T cells, from GKO$^{-/-}$ and GKO$^{+/-}$ mice, reduced CFU to a similar degree in spleen and liver (data not shown). These experiments strongly support the hypothesis that CD8 T cell-derived IFNγ is not required for transfer of antilisterial resistance by antigen-specific CD8 T cells.

CD8 T Cell-Derived IFNγ Does Not Enhance Transfer of Antilisterial Immunity

IFNγ-independent transfer of antilisterial immunity by relatively large numbers of in vitro propagated single antigen-specific CD8 T cells, provides evidence that CD8 T cell-derived IFNγ is not required for expression of immunity but does not accurately reflect the in vivo situation in which CD8 T cell responses to multiple L. monocytogenes antigens would be expected. To address this issue, we infected GKO$^{-/-}$ or GKO$^{+/-}$ mice with L. monocytogenes DP-L1942 and 7 days later transferred 4 × 10^7 or 8 × 10^7 CD4 T cell-depleted spleen cells from these animals into naive BALB/c mice, which were then challenged with 10 LD_{50} of L. monocytogenes 10403s. Under these conditions, transfer of antilisterial immunity has been shown to depend on CD8 T cells (Dunn and North, 1991b), which comprise <10% of the transferred cells after CD4 T cell depletion (data not shown). Although transfer of 4 × 10^7 L. monocytogenes immune spleen cells resulted in less immunity than transfer of 8 × 10^7 L. monocytogenes immune spleen cells, no IFNγ-dependent difference in the degree of immunity was detected (Figure 4). The spleens of DP-L1942-infected GKO$^{-/-}$ and GKO$^{+/-}$ mice contained similar percentages of CD8 T cells at 7 days after infection and exhibited similar levels of antigen-specific lysis after 5 days of in vitro restimulation with the LLO-expressing P815 transfectant (data not shown), suggesting that the number of antigen-specific CD8 T cells transferred from each strain was similar. Thus, the ability of CD4 T cell-depleted L. monocytogenes immune spleen cells to transfer antilisterial immunity is not enhanced by the ability of these cells to secrete IFNγ.

Transfer of Antilisterial Immunity by GKO$^{-/-}$-Derived Spleen Cells Does Not Depend on Recipient-Derived IFNγ

Transfer of antilisterial immunity, by GKO$^{-/-}$- and GKO$^{+/-}$-derived CD8 T cells to naive BALB/c mice occurs in an environment where natural immunity, mediated by NK cell-derived IFNγ, is active and contributing to the resolution of infection. Indeed, the possibility existed that immunity mediated by GKO$^{-/-}$-derived CD8 T cells in BALB/c mice could have resulted from activation of NK cells to up-regulate IFNγ production. To address this question, we performed adoptive transfer assays using GKO$^{-/-}$ hosts, an environment completely devoid of NK cell-derived IFNγ. CD4 T cell-depleted spleen cells (8 × 10^7) from DP-L1942-infected GKO$^{-/-}$ or GKO$^{+/-}$ mice were transferred to naive GKO$^{-/-}$ mice prior to infection with 1.6 × 10^3 CFU of L. monocytogenes 10403s, a level representing ~ 160× the LD_{50} for naive GKO$^{-/-}$ mice (Table 1). As shown in Figure 5, GKO$^{-/-}$-derived L. monocytogenes immune spleen cells mediate significant immunity, comparable to GKO$^{+/-}$-derived CD8 T cells, even in the absence of host-derived IFNγ. Similar reduction in CFU were observed in liver homogenates from these animals (data not shown). Depletion of 94% of CD8 T cells by antibody and complement prior to transfer of immune GKO$^{-/-}$ spleen cells reduced the degree of immunity transferred by 90% (data not shown). These experiments demonstrate that IFNγ is not required for CD8 T cell-mediated antilisterial immunity under adoptive transfer conditions.

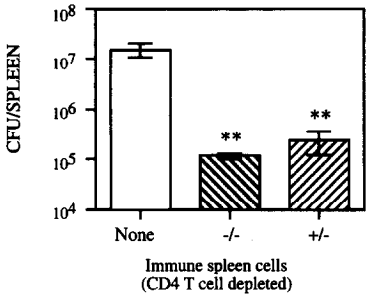

Figure 5. Recipient-Derived IFNγ Is Not Required for Adoptive Transfer of Antilisterial Resistance by GKO$^{-/-}$ CD8 T Cells

Spleen cells from GKO$^{-/-}$ or GKO$^{+/-}$ mice were obtained 7 days after infection with 2 × 10^6 CFU of DP-L1942. CD4 T cell–depleted (8 × 10^7) spleen cells were transferred into naive GKO$^{-/-}$ mice. These mice and control GKO$^{-/-}$ mice were infected with 1.6 × 10^3 CFU of 10403s. CFU/spleen and liver were determined 3 days later. Results are expressed as mean ± SD for groups of 3 mice. Double asterisks, $p < 0.025$ compared with control.

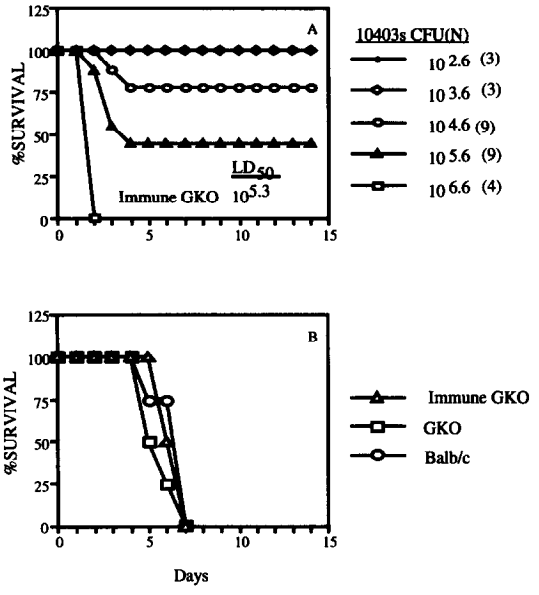

Figure 6. Vaccination of GKO$^{-/-}$ Mice with DP-L1942 Induces Specific Immunity in the Absence of IFNγ

(A) GKO$^{-/-}$ mice were infected with 2 × 10^6 CFU of DP-L1942. 32 days later groups (N) of animals were challenged with the indicated number of 10403s. Survival was monitored for 14 days. The LD_{50} in this experiment was $10^{5.3}$ CFU.

(B) Groups of 4 vaccinated GKO$^{-/-}$, naive GKO$^{-/-}$, and naive BALB/c mice were challenged by intraperitoneal injection of 100 CFU of S. typhimurium SL1344 (~5 LD_{50} for BALB/c mice). Survival was monitored for 14 days.

Vaccination of GKO$^{-/-}$ Mice with Attenuated L. monocytogenes Induces Specific Resistance to Virulent L. monocytogenes

The ability of CD8 T cells, derived from DP-L1942-infected GKO$^{-/-}$ mice, to transfer antilisterial immunity to naive mice in the absence of IFNγ suggested the possibility that vaccination of GKO$^{-/-}$ mice with attenuated L. monocytogenes may enhance resistance to infection. GKO$^{-/-}$ mice were vaccinated by infection with 1 × 10^6 L. monocytogenes DP-L1942 and 32 days later were challenged with various doses of virulent L. monocytogenes 10403s to assess the degree of resistance generated. Vaccinated GKO$^{-/-}$ mice exhibited a striking degree of resistance (LD_{50} of $10^{5.3}$ CFU, Figure 6A) compared with naive GKO$^{-/-}$ mice (LD_{50} of 10 CFU, Table 1). This resistance is specific, since DP-L1942-vaccinated GKO$^{-/-}$ mice, which resist ~20,000-fold more virulent L. monocytogenes than naive GKO$^{-/-}$ mice, exhibit the same incidence and kinetics of mortality after infection with an unrelated mouse pathogen, Salmonella typhimurium strain SL1344 (Hoiseth and Stocker, 1981), as do naive GKO$^{-/-}$ and BALB/c mice (Figure 6B). Thus, specific immunity to L. monocytogenes can be generated in the absence of IFNγ. In addition, the difference in resistance of naive GKO$^{-/-}$ mice and GKO$^{-/-}$ mice receiving a single injection with an attenuated L. monocytogenes strain demonstrates that vaccination-induced immunity can overcome the lack of a cytokine that is critical for resistance to acute infection.

Vaccination Induced Immunity in GKO$^{-/-}$ Mice Is Dependent on CD8 T Cells

To assess the effectors of vaccination-induced immunity, DP-L1942 vaccinated GKO$^{-/-}$ mice were depleted of specific T cell subsets by treatment with anti-CD4 MAb GK1.5 (Dialynas et al., 1983) or anti-CD8 MAb 2.43 (Sarmiento et al., 1980). GK 1.5 treatment reduced the number of CD4 T cells by >95%, as determined by FACs analysis of spleen cells from depleted and control mice. 2.43 treatment reduced the number of CD8 T cells by >97%. T cell subset depleted and control mice were then challenged with virulent L. monocytogenes 10403s on day 32 postvaccination. Depletion of CD4 T cells did not significantly alter the resistance of vaccinated GKO$^{-/-}$ mice to challenge with virulent L. monocytogenes (Figure 7A). In contrast, depletion of CD8 T cells diminished the resistance of vaccinated GKO$^{-/-}$ by at least two orders of magnitude (Figure 7B). CD8 T cell–depleted immune GKO$^{-/-}$ mice exhibit slightly more resistance to virulent L. monocytogenes than naive GKO$^{-/-}$ mice. This finding may result from expansion of the small number of remaining CD8 T cells in these mice in time to resist the number of organisms in the low dose challenge. Alternatively, other cell types, such as γ–δ T cells, may contribute to the resistance of vaccinated GKO$^{-/-}$ mice in the absence of IFNγ. These experiments demonstrate that DP-L1942 vaccination of GKO$^{-/-}$ mice primes a L. monocytogenes–specific immune response capable of resisting infection with high dose virulent L. monocytogenes.

Discussion

Lethal infection of H-2^d GKO$^{-/-}$ mice requires ~1000-fold fewer L. monocytogenes than the number required for lethal infection of mice with an intact IFNγ structural gene. These data confirm the importance of IFNγ in natural im-

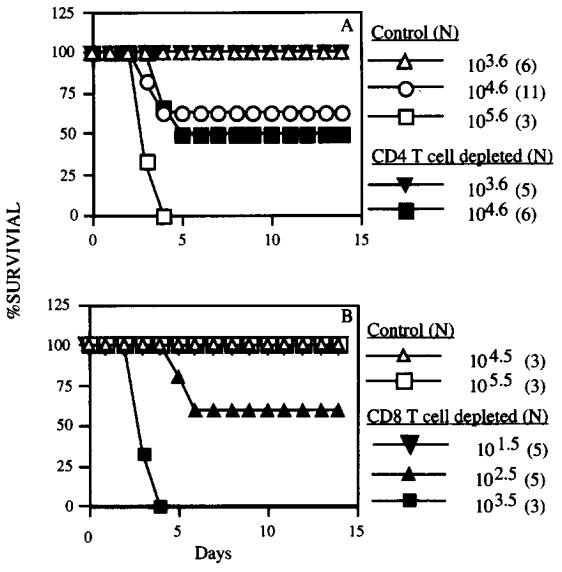

Figure 7. Vaccination-Induced Immunity in GKO$^{-/-}$ Mice Depends on CD8 T Cells

(A) GKO$^{-/-}$ mice were infected with 7 × 10^5 CFU of DP-L9142. Mice were subsequently depleted of CD4 T cells (closed symbols) or not (open symbols) and were challenged at 32 days postvaccination with indicated number of 10403s.

(B) GKO$^{-/-}$ mice were infected with 4 × 10^6 CFU of DP-L1942. Mice were subsequently depleted of CD8 T cells (closed symbols) or not (open symbols) and were challenged at 32 days postvaccination with the indicated numbers of 10403s. The degree of T cell depletion was monitored by flow cytometry. Animals were monitored for survival for 14 days. The number of animals in each group are indicated in parenthesis.

munity to acute L. monocytogenes infection demonstrated by earlier studies with neutralizing anti-IFNγ MAb (Buchmeier and Schreiber, 1985) and mice with targeted disruption of the IFNγ receptor gene (Huang et al., 1993) but also demonstrate the critical role IFNγ plays in resistance to acute infection. Consistent with a major role for IFNγ in resistance to intracellular bacterial and protozoan pathogens are reports that GKO$^{-/-}$ mice, expressing H-2^b MHC molecules, exhibit increased susceptibility to acute infection with Mycobacterium tuberculosis (Cooper et al., 1993; Flynn et al., 1993) and Leishmania major (Wang et al., 1994).

The availability of well-characterized attenuated L. monocytogenes mutants and GKO$^{-/-}$ mice suggested a strategy to localize the antimicrobial action of IFNγ to a particular stage of L. monocytogenes infection. The three orders of magnitude decrease in resistance to L. monocytogenes exhibited by GKO$^{-/-}$ mice, compared with wild-type mice, provides a window to characterize the virulence of L. monocytogenes mutants, which are attenuated for virulence in wild-type mice, in mice incapable of producing IFNγ. Conversion of the attenuated phenotype to virulence in the absence of a particular cytokine would indicate a role for that cytokine in containing the organism at the specific stage of infection identified by the mutation. In our initial experiments, we determined that GKO$^{-/-}$ mice resist infection with an *actA* mutant L. monocytogenes at a level comparable to mice with intact IFNγ genes. These data suggest that the role of IFNγ in resistance to L. monocytogenes infection may involve prevention of cell–cell spread, since IFNγ is not required for resistance to L. monocytogenes that are incapable of cell–cell spread. These data are in agreement with in vitro studies suggesting that IFNγ activation of a macrophage-like cell line prevents escape of the organism from the phagosome (Portnoy et al., 1989). One prediction from this hypothesis is that L. monocytogenes strains capable of limited cell–cell spread, such as the phospholipase mutants, should exhibit increased virulence in GKO$^{-/-}$ mice compared with wild-type mice. Further studies with other defined L. monocytogenes mutants in GKO$^{-/-}$ mice will directly test this hypothesis.

Although they are attenuated for virulence, *act A* mutant L. monocytogenes enter the cytoplasm of infected cells and are accessible to the MHC class I antigen presentation pathway. Indeed, infection of wild-type mice with *act A* mutant L. monocytogenes elicits a CD8 T cell response that can transfer antilisterial immunity to naive mice (Goossens and Milon, 1992). Since GKO$^{-/-}$ mice survive infection with *actA* mutant L. monocytogenes, we could use this strain to elicit L. monocytogenes–specific CD8 T cells that are incapable of producing IFNγ. GKO$^{-/-}$-derived CD8 T cell lines specific for epitopes derived from the secreted LLO or p60 molecules, transferred similar levels of immunity to naive BALB/c mice as CD8 T cell lines specific for the same antigens, derived from mice with intact IFNγ genes. These experiments directly demonstrate that CD8 T cell–derived IFNγ is not required for transfer of antilisterial immunity to naive mice and suggest that other effector mechanisms play a dominant role in this process. Consistent with this interpretation are recent studies indicating that CD8 T cells from perforin-deficient mice have reduced capacity to transfer antilisterial resistance (Kagi et al., 1994). Together, these studies support a role for direct lysis of infected cells by L. monocytogenes–specific CD8 T cells.

One consequence of this model is the requirement for accessory cells to destroy L. monocytogenes that are released from target cells after CD8 T cell–mediated lysis. Interestingly, two recent reports demonstrate that antibody-mediated depletion of neutrophils in L. monocytogenes immune mice inhibits the expression of specific immunity to rechallenge (Appleberg et al., 1994; Czuprynski et al., 1994). In combination, these experiments provide both direct and indirect support for the importance of CD8 T cell–mediated lysis of infected cells as an effector mechanism in immunity to L. monocytogenes. It should be pointed out, however, that these data do not rule out a contribution by other CD8 T cell–derived cytokines in the expression of antilisterial immunity. GKO$^{-/-}$ mice provide an experimental system devoid of IFNγ to address this question.

Our results demonstrate that IFNγ is a critical cytokine in natural immunity to acute L. monocytogenes infection. Natural immunity to L. monocytogenes, which requires neutrophils (Conlan and North, 1994; Czuprynski et al., 1994) as well as activated macrophages (Bancroft et al.,

1991), is thought to limit infection until the development of specific immunity. In this scenario, early IFNγ production by NK cells may play several roles. First, experimental evidence suggests that IFNγ-mediated macrophage activation is required to control the acute infection (Wherry et al., 1991), possibly by limiting cell–cell spread of the organism. Consistent with this evidence are studies showing that inhibitors of IFNγ-dependent nitric oxide generation exacerbate L. monocytogenes infection in vivo (Beckerman et al., 1993; Boockvar et al., 1994). Second, the well-documented effects of IFNγ on antigen presentation, including up-regulation of MHC class I and class II genes, *tap* genes, and *LMP* genes (Germain, 1994), may aid in development of specific immunity to L. monocytogenes. IFNγ-mediated up-regulation of antigen presentation functions may be most relevant when the acute infection approaches lethal level, where delays in the development of specific immunity could result in lethal infection. Thus, IFNγ may play multiple roles in resistance to acute L. monocytogenes infection.

In contrast with the critical role of IFNγ in natural immunity to L. monocytogenes, we were unable to detect a requirement for IFNγ in specific immunity to infection. This result is most striking in light of the many effects on the immune system mediated by IFNγ in addition to macrophage activation (Farrar and Schreiber, 1993). Although IFNγ may aid in development of specific immunity to L. monocytogenes, our data demonstrates that it is not required for vaccination-induced immunity in GKO$^{-/-}$ mice. These studies also demonstrate that IFNγ-mediated macrophage activation and up-regulation of antigen-presenting functions are not required for the expression of specific immunity in an immunized host. The effects of CD8 T cells in adoptive transfer assays are thought to occur early after challenge infection, at a stage where the majority of L. monocytogenes may be confined to macrophages (Dunn and North, 1991b). However, L. monocytogenes also infects cells such as hepatocytes. The ability of hepatocytes and hepatocyte-like cell lines to exhibit antibacterial activity after cytokine treatment is somewhat controversial (Gregory and Wing, 1993; Wood et al., 1993); however, these cells are clearly not as bactericidal as activated macrophages (Gregory et al., 1992). Thus, the ability to secrete IFNγ may not help CD8 T cells in dealing with L. monocytogenes–infected nonmacrophage cells such as hepatocytes, which are a major site of L. monocytogenes replication in the acutely infected animal (Gregory et al., 1992). In addition, the ability of GKO$^{-/-}$-derived L. monocytogenes immune CD8 T cells to transfer immunity to naive GKO$^{-/-}$ hosts, as opposed to wild-type hosts, rules out an indirect effect of CD8 T cells to increase IFNγ secretion by NK cells. If CD8 T cell–derived IFNγ played any role in the transfer of antilisterial resistance to naive hosts, it was too subtle to detect in our experimental system.

The adoptive transfer assay of L. monocytogenes–specific CD8 T cells has been criticized as to relevance with regard to the mechanism by which L. monocytogenes is cleared from the acutely infected host (Dunn and North, 1991b). However, it is a relevant model system to analyze the consequences of infection in a previously immunized host (Harty and Bevan, 1992). To extend our findings, we vaccinated GKO$^{-/-}$ mice with a single injection of the *actA* mutant L. monocytogenes strain and asked whether these mice become immune to challenge with virulent L. monocytogenes. Strikingly, vaccinated GKO$^{-/-}$ mice developed as much as 20,000-fold increased specific resistance to virulent L. monocytogenes compared with naive GKO$^{-/-}$ mice. This immunity appeared to be primarily due to the actions of CD8 T cells, although contributions of other effector cells have not been experimentally ruled out. Thus, specific immunity to L. monocytogenes can be generated in the absence of IFNγ and does not require IFNγ as an effector molecule.

Our experiments demonstrate that IFNγ is a critical mediator of natural immunity to acute L. monocytogenes infection but find no obvious requirement for IFNγ in specific immunity to infection. The ability of GKO$^{-/-}$ mice to develop resistance to virulent L. monocytogenes, after infection with an attenuated L. monocytogenes strain, demonstrates that vaccination-induced immunity can overcome the lack of a cytokine, which is critical to resistance to acute infection. These studies suggest an untapped potential of cytokine and cytokine receptor knockout mice as models of immune deficiency to learn whether an immune response can be generated that overcomes the specific dysfunction. Such studies may impact vaccine strategies for immunocompromised individuals.

Experimental Procedures

Mice

BALB/c (H-2^d) mice were obtained from Jackson Laboratories (Bar Harbor, Maine). GKO$^{-/-}$ mice, originally generated on the 129/Sv (H-2^b) background (Dalton et al., 1993), were bred to BALB/c mice and backcrossed four times to generate H-2^{dd} GKO$^{-/-}$ mice, which were then maintained by brother–sister mating. Mice containing the disrupted gene were identified by Southern blot analysis as described (Dalton et al., 1993). GKO$^{+/-}$ mice were generated from GKO$^{-/-}$ mice by backcross to BALB/c.

Cell Lines, Antibodies, and Flow Cytometry

P815 cells are a DBA/2 (H-2^d)-derived mastocytoma tumor cell line. PHem3.3 (Harty and Bevan, 1992) cells are P815 cells transfected with the known CD8 T cell antigen LLO (Pamer et al., 1991). pP60.3 cells (Harty and Pamer, 1995) are P815 cells transfected with the known CD8 T cell antigen p60 (Pamer, 1994). P815 cells and transfectants were maintained as described (Harty and Bevan, 1992).

In vivo T cell subset depletion was performed with anti-CD4 MAb GK1.5 (Dialynas et al., 1983) and anti-CD8 MAb 2.43 (Sarmiento et al., 1980), which were purified from culture supernatants using protein G affinity chromatography as recommended by the manufactured (Pharmacia). Protein concentrations were determined using the bicinchoninic acid assay (Pierce). In vivo T cell subset depletion was carried out by intraperitoneal injection of 0.3 mg of MAb on days 28–30 postvaccination. T cell subset depletion was >95% in both cases. In vitro T cell subset depletion was carried out with immunoglobulin M (IgM) MAbs RL-172 (anti-CD4) or 3.168 (anti-CD8) and rabbit complement as previously described (Harty et al., 1992). CD4 T cell depletion was >97%. CD8 T cell depletion was >94%. Depletion of T cell subsets was monitored by staining with fluorescein isothiocyanate–conjugated anti-CD8 MAb 53.6–7 (Sigma) and phycoerythrin-conjugated anti-CD4 MAb H129.19 (Sigma). Cells were analyzed on a FACScan (Becton Dickinson) with Lysis II software. Cells were also stained with fluorescein isothiocyanate–conjugated goat anti-rat antibody to detect residual rat antibodies, which could block detection of CD4 or CD8 staining.

Bacteria and Immunizations

Listeria monocytogenes strains 10403s and DP-L1942 were gifts of D. Portnoy, University of Pennsylvania, and were grown as previously described (Harty and Bevan, 1992), except that streptomycin at 50 μg/ml was added to the medium. L. monocytogenes strain 10403s has an LD_{50} of $10^{4.1}$ CFU after intravenous injection of BALB/c mice (Bishop and Hinrichs, 1987), whereas DP-L1942, which contains an in-frame deletion in the *actA* gene, has an LD_{50} of $10^{7.3}$ CFU (Brundage et al., 1993). Salmonella typhimurium strain SL1344 (Hoiseth and Stocker, 1981) was the gift of B. Jones, University of Iowa, and was grown in Luria–Bertani broth. S. typhimurium SL1344 has an LD_{50} of $10^{1.3}$ CFU after intraperitoneal injection of BALB/c mice (Hoiseth and Stocker, 1981). For immunization of $GKO^{-/-}$ mice, 0.7–4 × 10^6 CFU of DP-L1942 were injected intravenously. Animals were challenged 32 days later with the indicated numbers of L. monocytogenes or S. typhimurium and LD_{50} were calculated as described (Portnoy et al., 1988). Actual numbers of bacteria injected were determined by plating aliquots of relevant dilutions.

Peptides and IFNγ Detection

Synthetic peptides representing the known H-$2K^d$-restricted epitopes LLO 91–99 (Pamer et al., 1991) and p60 217–225 (Pamer, 1994) were dissolved in phosphate-buffered saline and used at the indicated concentrations. Antigen-specific IFNγ production by CD8 T cells was determined by enzyme-linked immunosorbent assay (ELISA) with a kit from Genzyme as previously described (Harty et al., 1992).

CD8 T Cell Lines and Chromium Release Assays

$GKO^{-/-}$ and $GKO^{+/-}$ mice were infected by intravenous injection of $10^{4.0}$ CFU of DP-L1942. Spleen cells from these mice were restimulated in vitro 7 days later with irradiated PHem3.3 (15,000 rads), to elicit LLO-specific CD8 T cell lines, or irradiated pP60.3 to elicit p60-specific CD8 T cells lines. CD8 T cell lines were generated and maintained using the cell numbers and culture conditions previously described (Harty and Bevan, 1992). Long-term CD8 T cell lines were maintained in the presence of 5% Rat concanavalin A supernatant and 50 mM α-methyl mannoside as described (Harty and Bevan, 1992).

For chromium release assays, target cells (1 × 10^6) in 300 μl RP10 were labeled with 100 μCi ^{51}Cr sodium chromate for 1 hr at 37°C. After washing, 10^4 target cells were incubated with serial dilutions of effector cells in 200 μl of RP10 in round-bottomed 96-well plates. After 3–4 hr incubation at 37°C, 100 μl supernatant was collected and specific lysis was determined as: percent specific lysis = 100 × ([release by CTL − spontaneous release] / [maximum release − spontaneous release]). Spontaneous release in the absence of CTL was <10% in all experiments.

Adoptive Transfer Assays

The capacity of CD8 T cell lines to transfer antilisterial resistance was determined as previously described (Harty and Bevan, 1992). In brief, $GKO^{-/-}$- or $GKO^{+/-}$-derived CD8 T cell lines specific for p60 or LLO were harvested at day 7–9 after in vitro restimulation and were washed two times with phosphate-buffered saline. The indicated numbers of CD8 T cells were injected intravenously into naive BALB/c mice in a volume of 0.2 ml. These mice and uninjected control mice were infected intravenously with ~10^5 CFU (~10 LD_{50}) L. monocytogenes strain 10403s 1 hr later. CFU/spleen and liver were determined 3 days after infection. In other experiments, L. monocytogenes immune spleen cells were obtained from DP-L1942-infected $GKO^{-/-}$ and $GKO^{+/-}$ mice at 7 days postinfection. L. monocytogenes immune spleen cells were depleted of CD4 T cells or CD8 T cells prior to transfer to naive BALB/c or $GKO^{-/-}$ mice. $GKO^{-/-}$ mice were infected with 1.6 × 10^3 CFU (~160 LD_{50}). Transferred spleen cells contained less than 10 CFU of DP-L1942. Data is expressed as mean CFU ± SEM. Student's t test was used to determine the statistical relationship between groups.

Acknowledgments

Address correspondence to J. T. H. The authors wish to thank D. Dalton, T. Stewart, and Genentech, Incorporated, for providing the $GKO^{-/-}$ mice for these experiments. We wish to thank D. Portnoy for providing L. monocytogenes strain DP-L1942 prior to publication and for stimulating discussion; B. Jones for assistance with the Salmonella experiments; and B. Jones and C. Lutz for critical review of the manuscript. The excellent technical assistance of Ms. A. Vivado is gratefully acknowledged. This work was supported by the National Institutes for Health and The Howard Hughes Medical Institute. J. T. H. is supported by an Arthritis Investigator Award and the Roy J. Carver Charitable Trust.

Received March 16, 1995; revised April 20, 1995.

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